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A FLUORESCENCE STUDY OF THE BINDING PROPERTIES OF THE SYNTHETASE FROM ESCHERICHIA COLI

Geoffrey R. Penzer, Edward L. Bennett,
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Isoleucyl-tRNA-synthetase

A fluorescence study of the binding properties of the synthetase from *Escherichia coli*.

By Geoffrey R. Penzer, Edward L. Bennett and Melvin Calvin

Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory, and Department of Chemistry, University of California, Berkeley, California 94720

Running title: Fluorescence and binding properties of isoleucyl-tRNA-synthetase.

Proofs to: Dr. G. R. Penzer
Department of Chemistry
University of York
Heslington
York YO1 5DD
England

Subdivision 2 (protein chemistry - enzymology - physical chemistry)

Geoffrey R. Penzer

Department of Chemistry

University of York

Heslington

York YO1 5DD

England

Edward L. Bennett and Melvin Calvin

Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory

Department of Chemistry

University of California

Berkeley, California 94720, U.S.A.

Abbreviation: $tRNA^{Ile}$ - L-isoleucine accepting tRNA

Enzyme: isoleucyl-tRNA-synthetase or L-isoleucine:tRNA ligase (AMP) - EC 6.1.1.5

Summary

Fluorescence properties of purified isoleucyl-tRNA-synthetase isolated from E. coli B have been studied. No changes in the quantum yield, energy or polarisation of the emission were detected in the presence (either individually or in combinations) of the substrates and cofactors required for activation of L-isoleucine.

In 2.5 M urea enzyme activity and intrinsic fluorescence intensity (at 340 nm) each decrease with time, showing similar kinetics and rate constants. The rate of this decay is reduced in the presence of ligands which can bind to the enzyme and the effect has been used to measure dissociation constants for enzyme-ligand complexes. Values have been obtained for the complexes between enzyme and L-isoleucine (K_{diss} = 2.5 x 10^{-5} M), L-valine (K_{diss} = 3.0 x 10^{-4} M), ATP (K_{diss} = 1.5 x 10^{-4} M) and PP_i (K_{diss} = 2.0 x 10^{-4} M) at 25°. The effects of ionic strength, and the temperature dependence and urea concentration dependence of L-isoleucine binding have also been studied. Magnesium ions, which are required for catalysis, do not greatly affect the binding of single substrates, but changes are seen in the

presence of ATP and L-isoleucine together. The magnesium ion concentration dependence of this effect (half-point about 2×10^{-4} M) and the equilibrium constant for L-isoleucine activation (2×10^{-6} M) have both been measured.

The reliability of the methods has been discussed. Results have been interpreted in terms of current theories of amino acid activation. The binding parameters are sufficient to explain the stability of enzyme bound L-isoleucyladenylate without invoking conformation changes. This is consistent with the absence of substrate induced fluorescence changes. Magnesium effects are explained in terms of reduced electrostatic repulsion between reactants bearing like charges.

The reactions catalysed by isoleucyl-tRNA-synthetase are usually represented by the following equilibria:

a) Enzyme + ATP + Ile $\overset{Mg^{2+}}{\rightleftharpoons}$ Enzyme-AMP-Ile + PP_i

b) Enzyme-AMP-Ile + $tRNA^{Ile}$ $\rightleftharpoons$ Enzyme + AMP + $tRNA^{Ile}$-Ile

This is certainly an oversimplification as equilibria not represented in equations a and b are set up [1,2]. Alternatively there is the suggestion that tRNA charging proceeds by a concerted mechanism which does not involve the complex (enzyme-AMP-Ile) formed in reaction a [3]. Whatever the detailed mechanism it is generally accepted that isoleucyl-tRNA-synthetase recognises specifically L-isoleucine and $tRNA^{Ile}$. L-Valine is a possible but less good substrate than L-isoleucine in reaction a (commonly called the PP_i exchange reaction because of the assay method used, or the amino acid activation reaction), but it cannot subsequently be transferred to tRNA [4].

Cole and Schimmel have undertaken a kinetic investigation of the exchange reaction which has led them to postulate the formation of binary complexes between isoleucyl-tRNA-synthetase and each of

L-isoleucine, ATP and PP_i [2]. They also suggest that the observed dependence of exchange rate on $[Mg^{2+}]$ occurs because only the mono-magnesium complexes of ATP and PP_i can react [5]. It is not clear how large a deviation from the proposals would have been detected by their experimental methods. Yarus and Berg, studying interactions between isoleucyl-tRNA-synthetase and $tRNA^{Ile}$, have found that the rates at which complexes between enzyme and tRNA form and dissociate are both enhanced in the presence of L-isoleucine [1,6]. They explain their results on the hypothesis that isoleucyl-tRNA-synthetase adopts several conformations during a single catalytic cycle [1]. A problem in comparing and interpreting the results of these and other groups is that the physical conditions used are often widely different.

In this work we have studied the intrinsic fluorescence of isoleucyl-tRNA-synthetase. The intensity of fluorescence decays in 2.5 M urea at a rate which depends on the concentrations of substrates also present. Measurement and analysis of the decay rates have enabled us to calculate dissociation constants for complexes of the enzyme.

The method, which is novel, is simple and requires only small amounts of enzyme (a dissociation constant can be obtained with 50-100 μg of isoleucyl-tRNA-synthetase).

There have been 4 main aims in the work we describe: 1) to substantiate the existence of the complexes proposed by Cole and Schimmel by a method less equivocal than deduction from complex kinetics; 2) to study the effects of Mg^{2+} on the interaction of ligands with isoleucyl-tRNA-synthetase; 3) to measure the effects of temperature and ionic strength on some of the binding interactions; 4) to construct a plausible mechanism for activation of L-isoleucine by isoleucyl-tRNA-synthetase.

MATERIALS

E. coli B cells (late log) were purchased from Miles Laboratories Inc., Elkhart, Indiana, or General Biochemicals, Chagrin Falls, Ohio. Isoleucyl-tRNA-synthetase was prepared from E. coli B by the method of Baldwin and Berg [7] with only minor modifications. The final product, which showed a single band in gel electrophoresis and always

had a specific activity of 500-600 units/mg, was stored at -15° at about 1 mg/ml in 0.02 M potassium phosphate (pH 7.5) containing 0.001 M glutathione. It was routinely assayed by the PP_i exchange method [8] in an incubation mixture of the following composition: 0.1 M Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM KF, 10 mM 2-mercapto-ethanol, 2 mM Na_2ATP, 2 mM L-isoleucine or L-valine, 2 mM $Na_4\ ^{32}PP_i$ (activity 4,000-10,000 cpm/μmol) and enzyme in a total volume of 1 ml.

$^{32}PP_i$ was prepared by pyrolysis of $Na_2H^{32}PO_4$ (International Nuclear and Chemical Corp., Irvine, California) by the method of Bergmann et al. [4].

Amino acids, ATP and urea were A grade products of Calbiochem, Los Angeles, California. Trizma base was obtained from Sigma Chemical Co., St. Louis, Missouri. All other compounds were supplied by J. T. Baker Chemical Co., Phillipsburg, New Jersey. Solutions were all prepared in twice distilled water.

METHODS

Experimental procedure

Fluorescence measurements were made with a Hitachi MPF-2A

fluorescence spectrophotometer with the temperature of its sample block controlled by circulating water from a thermostat bath. Polarisation measurements were made using polarising filters (Hitachi) in both excitation and emission beams. The spectra obtained are uncorrected for either photomultiplier response or the emission spectrum of the xenon source.

An experimental technique which has proved satisfactory for measuring the rates of fluorescence decay of isoleucyl-tRNA-synthetase is as follows. A stock solution of isoleucyl-tRNA-synthetase (about 3 µg/ml and sufficient volume for a day's measurements) is prepared in 0.1 M Tris-HCl at pH 8.0, and kept at 0°. 70 g urea are dissolved in 100 ml 0.1 M Tris-HCl and the pH is adjusted to 8.0. After correction for volume changes when urea dissolves this solution is found to be 7.5 M urea. It is stored at the temperature of the fluorometer sample block. A series of solutions of the prospective ligand in Tris-HCl are prepared to cover a range of concentrations; pH is adjusted to 8.0 if necessary.

To make a measurement, 2 ml of isoleucyl-tRNA-synthetase solution is transferred to a test tube which is placed in a water bath at the temperature of the sample block for 5 to 10 min. 0.1 ml of ligand solution is added. Finally 1 ml of 7.5 M urea is mixed with the enzyme, shaken vigorously for 5 sec and transferred to a 1 cm fluorometer cuvette which is immediately placed in the instrument. Decay of fluorescence is followed at 340 nm (exciting at 280 nm) for 10 to 20 min. Bandwidths were in the range 5 to 10 nm. Sometimes anomalies in the fluorescence decay appear during the first minute (perhaps due to temperature effects or to imperfect mixing) but after that the decay is exponential. Usually each rate was measured 3 times and the average of the results taken.

The best way to obtain rates from the data is to measure F (fluorescence intensity)$_{t=\infty}$/F$_{t=0}$ for one or two samples (Fig. 1) and to use this ratio to calculate $F_{t=\infty}$ for each run. Now F_t-$F_{t=\infty}$ can be plotted against time on semi-log graph paper (see Results section) and the gradient gives the rate of fluorescence decay (Fig. 2). The reproducibility for identical runs was ± 15%.

Theory

In the experiments that we describe the isoleucyl-tRNA-synthetase concentration was always much lower (1% or less) than the total ligand concentration. We have analysed our data on the simplest assumptions--that free ligand concentration differs negligibly from total ligand concentration and that all binding sites (if there are more than 1 per enzyme molecule) are homogeneous. There is considerable evidence for a single catalytic site per molecule of isoleucyl-tRNA-synthetase [1,9] and no evidence for binding of substrates for amino acid activation at non-catalytic sites.

Consider the equilibrium Enzyme (E) + ligand (L) $\rightleftharpoons$ E-L. We assume that the initial rate of fluorescence decay (R) of a given equilibrium mixture is compounded of only 2 rates, R_a (= $k_a[E]$) and R_b (= $k_b[E\text{-}L]$). The effect of multiple bonding of ligands, if it occurs, is ignored. The dissociation constant of the complex (K_L) is defined by $K_L = [E][L]/[E\text{-}L]$. $[E] + [E\text{-}L] = [E]_o$ (the total enzyme concentration) at zero time. Thus $R = R_a + R_b = k_a[E] + k_b([E]_o - [E])$.

R_o (rate of inactivation when [L] = 0) = $k_a[E]_o$

and $[E] = K[E]_o/(K + [L])$

Therefore $1/(R_o-R) = 1/\{[E]_o(k_a-k_b)\} + K/[L]\{[E]_o(k_a-k_b)\}$

So by plotting the reciprocal of the difference between the initial rate of decay of fluorescence in the absence and presence of ligand against 1/[L] a straight line should be obtained. K_L = [L] when $1/(R_o-R) = 2/(R_o-R_\infty)$, where R_∞ is the rate of decay at infinite [L].

If our assumptions are wrong the data will not fit this simple analysis.

RESULTS

Fluorescence spectra of isoleucyl-tRNA-synthetase

Excitation and emission spectra of isoleucyl-tRNA-synthetase are shown in Fig. 3. The shape of the emission spectrum is the same (± 1%) whether excitation is at 260 nm or at 280 nm. While there are negligible differences between spectra obtained in 0.02 M potassium phosphate (pH 7.5) and those in 0.1 M Tris-HCl (pH 8.0) there is a small shift of the emission maximum to longer wavelengths (2 to 5 nm) in 0.05 M potassium phosphate (pH 5.5). The quantum yield of fluorescence is

sensitive to temperature (Table 1) but the shape of the emission spectrum is unchanged from 4° to 35°.

Polarisation of fluorescence values [10] of isoleucyl-tRNA-synthetase (corrected for instrumental polarisation where necessary [11]) are given in Table 2 (lines A, B and C). The numbers are typical of those found for proteins which contain tryptophan [12] (isoleucyl-tRNA-synthetase contains 25 tryptophans/molecule, molecular weight 112,000 [7]).

Stability of spectra

Isoleucyl-tRNA-synthetase is sensitive to aerobic oxidation [13] so it is not surprising that over several hours the fluorescence of a sample changes. The rate of change (a fall of intensity) is greater at room temperature than at 10°. 1 mM glutathione and 10% glycerol each protect against the decay which is also reduced if solvents are deaerated (by evacuation) before use.

Effects of ligands on the fluorescence spectra

There were no changes in either the emission and excitation spectra or the fluorescence polarisation (Table 2) of isoleucyl-tRNA-

synthetase on adding any of the species listed in Table 3. As well as compounds which normally interact with the enzyme during PP_i exchange these species include a non-reacting amino acid (L-leucine), a high salt concentration, and glutathione at a higher concentration than is present in any of the solutions normally studied. Any fluorescence changes greater than 2% would have been detected. ATP absorbs light in the same region as isoleucyl-tRNA-synthetase so adding 2 mM Na_2ATP causes a reduction in the fluorescence intensity of the enzyme. The shape of the emission spectrum and the polarisation of fluorescence (Table 2) are unaltered. At long excitation wavelengths where absorption by ATP is negligible, the excitation spectrum is also unchanged.

Magnesium acetate, at concentrations above 1 mM, quenches the fluorescence of isoleucyl-tRNA-synthetase, but this is probably by a collisional process for the following reasons. At 10° the shape of the emission spectrum is unchanged on adding 10 mM magnesium acetate. The quenching increases as the temperature is raised (Table 1). 13 mM magnesium chloride is about 30% as good a quencher as 13 mM magnesium

acetate showing that a major part of the quenching is caused by acetate rather than Mg^{2+} ions.

Effects of urea

The fluorescence intensity of isoleucyl-tRNA-synthetase falls more rapidly in 2.5 M urea than it does in buffer alone. The solution remains clear, and shaking the cuvette does not alter the emission intensity so the change in fluorescence is caused by a reduction of the quantum yield of soluble protein and not by precipitation or changes in light scattering due to sedimentation. At 10° the half-time for decay (about 75 min) is 5 to 10 times longer than it is at 25°. The reproducibility of the measurement for 2 sets of identical runs is shown in Fig. 4. At 25° the rate of fluorescence decay rises sharply above pH 8.0, but it is not much affected by ionic strength (Table 4). Lower concentrations of urea cause considerably lower rates of decay (Table 5).

The time course of fluorescence decay correlates with the loss of enzyme activity (measured by PP_i exchange). In each case the kinetics are close to first order for at least the first 80% of reaction (Fig. 2). The fluorescence emission of the product after decay has proceeded to

completion has a reduced quantum yield and its maximum shifted about 10 nm to longer wavelengths than the native enzyme. This suggests that the tryptophans of denatured isoleucyl-tRNA-synthetase are in a more aqueous environment than those of the native enzyme.

Binary complexes

The data in Fig. 4 show that L-isoleucine protects isoleucyl-tRNA-synthetase from fluorescence decay in 2.5 M urea. This offers a method for measuring the binding constant of L-isoleucine (and, it turns out, of other ligands) to the enzyme. The suggested procedure is to measure rates of fluorescence decay at a series of ligand concentrations and to deduce from these values a binding constant. The form of the dependence of fluorescence decay rate on ligand concentration is governed by the number and heterogeneity of the binding sites for ligand on the enzyme. We have analysed our data using the simplest assumptions (see Methods section) and have found no significant deviations from predicted behavior.

At 25° the rate at which isoleucyl-tRNA-synthetase fluorescence decays in 2.5 M urea is reduced by each of L-isoleucine, L-valine, Na_2ATP and Na_4PP_i. Rates of decay greater than 85% of the unprotected rate have

been measured for 3.3 mM magnesium acetate, 1.0 mM ADP, 0.3 mM L-alloisoleucine, 0.3 mM D-alloisoleucine, 0.3 mM DL-alanine, 0.33 M KCl and 0.02 M potassium phosphate (pH 8.0). This suggests that none of these compounds binds strongly to the enzyme under the conditions tested. The dissociation constants for those complexes which have been detected are given in Table 6. In each case $1/(R_0-R):1/[L]$ plots were linear, though the precision of the data is inadequate to exclude the possibility of of small deviations (Fig. 5). Each measurement has been repeated on different preparations of the enzyme. No deviations outside the experimental error were detected.

The slopes and intercepts in Fig. 5 have been calculated by regression analysis without use of weighting factors although measurements at high ligand concentrations are the more accurate. In b) the point at 3.3×10^{-5} M L-valine was ignored for this reason, and because measurements at 1.7×10^{-5} M L-valine give $1/D \simeq \infty$. In c) at 3×10^{-6} M ATP $1/D \propto \infty$ and in d) at 1.7×10^{-5} M PP_i $1/D \propto \infty$. For this reason it is likely that the regression (r) line calculated for d) using all 6 points should be replaced by the line s. In each case we have applied a t-test to the

data to calculate the 95% confidence limit in the regression coefficient [14]: this is generally within a ± 50% error.

In an effort to discover whether 2.5 M urea causes large changes in the binding of L-isoleucine to isoleucyl-tRNA-synthetase we have measured the dissociation constant at lower urea concentrations (Table 6). The slower decay rates are harder to measure accurately, and so the K-values obtained in more dilute urea are less precise, but it is clear that the changes caused by urea (if any) are much less than an order of magnitude.

Multiple binding under non-catalytic conditions

Magnesium ions are essential for amino acid activation by isoleucyl-tRNA-synthetase [15] so their effects on the binding of L-isoleucine, ATP and PP_i have been tested. The results (Table 7) show that there are no major changes (an order of magnitude or more in K_L) in the presence of 3.3 mM Mg^{2+}, a concentration which is sufficient for catalytic activity. It is also shown, in Table 7, that a considerable increase in ionic strength (0.33 M KCl) scarcely perturbs the binding of these ligands.

Table 8 shows the effects of adding 2 of L-isoleucine, ATP and PP_i to isoleucyl-tRNA-synthetase simultaneously. Protections by ATP and PP_i are competitive, but either ATP or PP_i increases the protection exerted by L-isoleucine alone. No Mg^{2+} was added, so even in the presence of L-isoleucine and ATP catalysis cannot occur. The dissociation constant for L-isoleucine in the presence of 1.0 mM Na_2ATP has been measured in the usual way. Its value, 2.5×10^{-4} M (Fig. 6) shows that the binding between L-isoleucine and enzyme is less strong when ATP is bound as well.

Binding under Catalytic Conditions

When 1.7 mM magnesium acetate is added to a solution of isoleucyl-tRNA-synthetase containing 1.0 mM ATP, and the whole mixture is titrated with L-isoleucine the rate of fluorescence decay changes by less than 15%. This is true even under conditions where it is known that the enzyme is active in the PP_i exchange assay, and where in the absence of Mg^{2+} some protection is observed (Table 9). It means that the protection of isoleucyl-tRNA-synthetase exerted by formation of the enzyme-AMP-Ile complex is similar to the protection in enzyme-ATP. Thus at lower L-isoleucine and ATP concentrations, which alone exert very little

protection, addition of 1.7 mM Mg^{2+} does cause a significant reduction in the rate of fluorescence decay (Table 9). Using this effect it has been possible to measure the equilibrium constant of the PP_i exchange reaction (K_{equ} = [enzyme-AMP-Ile] [PP_i] / [enzyme] [ATP] [Ile). In some experiments there were equal low concentrations of Na_2ATP and Na_4PP_i with varying amounts of L-isoleucine, and in others the L-isoleucine concentration was small but constant while that of Na_2ATP was varied (Fig. 7). The K_{equ} values obtained were 1.5×10^{-6} M (ATP varied) and 7×10^{-6} M (L-isoleucine varied). Of these the former should be the more accurate because under the experimental conditions of varying L-isoleucine there is a greater concentration of enzyme-Ile than when L-isoleucine concentration is constant but small. This is reflected in a slightly higher maximum protection, and a slightly higher K_{equ}. This effect only explains part of the discrepancy between K_{equ} values: the remainder is within the bounds of experimental error which in this case (where extents of protection are small) is a factor of 2. The rate of decay at maximum protection is 65-70% of the unprotected rate.

To learn about the effect of Mg^{2+} we have titrated isoleucyl-tRNA-synthetase with magnesium acetate in the presence of 5×10^{-6} M ATP and 10^{-6} M L-isoleucine (Fig. 8). The concentration of magnesium acetate which gives half the maximum effect is 1 to 3×10^{-4} M.

DISCUSSION

Properties of isoleucyl-tRNA-synthetase

Stabilisation of L-isoleucyladenylate. We discuss our results in terms of the equation a (see introduction) which describes amino acid activation. It is not yet possible to consider a more detailed chemical mechanism because of lack of experimental evidence.

Enzymes, in general, have two functions: catalysis and selection. When concerned with a reaction whose rate is negligible in the absence of a catalyst the selection can be achieved by providing a catalytic mechanism for only the required substrate. In the case of amino acid activation, however, the problem is not only a kinetic one. The free energy of formation of an aminoacyladenylate (the species probably formed during amino acid activation [9]) from amino acid and ATP in aqueous solution is positive ($\Delta G^{o} \approx +2$ Kcal/mol, estimated from data in [19]). A major function of an amino acid activating enzyme is thus

to stabilise the adenylate of only the amino acid for which it is specific. One would not, therefore, expect the behaviour of the enzymes as a class to be homogeneous. The nature of forces stabilizing the adenylates of glycine or one of the more polar amino acids are likely to be different from those acting on amino acids with large non-polar side chains. The stabilisation presumably comes from binding interactions which are thus of special interest in these enzymes.

It is possible to estimate free energies of formation for several of the complexes of isoleucyl-tRNA-synthetase (Table 10). Though not very precise the values show that the standard free energy of binding L-isoleucyladenylate to the enzyme is similar to (and if anything less negative than) the sum of the binding energies for L-isoleucine and ATP individually. The dissociation constant calculated for the adenylate-enzyme complex is 10^{-8} to 10^{-7} M. This can be compared with the inhibition constant (5×10^{-8} to 7×10^{-9} M) measured for the close analogue, L-isoleucinyladenylate [20]. This means that from a thermodynamic point of view it is unnecessary to postulate conformational changes in

isoleucyl-tRNA-synthetase during activation of L-isoleucine. The energy changes observed are consistent with substantially unchanged binding sites for both L-isoleucine and ATP.

The Mg^{2+} effect. In the absence of Mg^{2+} the presence of ATP reduces the strength with which L-isoleucine binds to the enzyme. The fact that L-isoleucine binds at all means that the amino acid and ATP binding sites are distinct from each other. The fact that it binds less strongly when ATP is already bound means that there is repulsion between the ligands. The cause of this could be an electrostatic interaction between carboxyl and phosphate groups. If this suggestion is correct, the function of Mg^{2+} (which is now all that is necessary to trigger catalysis) may be to reduce the electrostatic repulsion by forming the monomagnesium complex of ATP and thus to potentiate the formation of L-isoleucyladenylate.

If this model for the mechanism of the Mg^{2+} effect is correct, one predicts that the Mg^{2+} titration curves for adenylate and for monomagnesium ATP formation are the same. The half-point of the Mg^{2+} dependence of adenylate formation estimated from Fig. 9 is 1 to 3 x 10^{-4} M (this

corresponds to a pK of about 3.7). The pK_{Mg}-values which have been measured for binding to free ATP are in the range 3.47 to 4.22 at 25° [21]. The pK_a of ATP is 6.5 to 7.0 [21] so at pH 8.0 about 5% of ATP is protonated and binds Mg^{2+} much less strongly. Perfect agreement between the Mg^{2+} dependence of catalysis and the formation of unbound $MgATP^{2-}$ is unlikely because binding ATP to the enzyme probably modified its properties. The figures quoted are certainly consistent with the proposed role of Mg^{2+} in catalysis. The data in Table 7 also lend support by showing that the effect of Mg^{2+} is not to alter in some gross way the binding of L-isoleucine or ATP to the enzyme.

The reversal of adenylate formation (induced by PP_i) seems to require Mg^{2+}, though not, perhaps, so critically as the forward reaction [9]. The effects of 3 mM Mg^{2+} on PP_i binding are small (Table 7) and again the cause of the Mg^{2+} effect may be its ability to reduce electrostatic repulsion between the reactants.

A consequence of the proposed action of Mg^{2+} during amino acid activation by isoleucyl-tRNA-synthetase is that although specific activities with L-isoleucine and L-valine as substrates are different,

the dependence of PP_i exchange rate on $[Mg^{2+}]$ should be the same in each case. We have found this to be so (Fig. 10). Ca^{2+} can replace Mg^{2+} for $tRNA^{Ile}$ charging [22], and we have also found that Mn^{2+} is effective in the PP_i exchange assay though with a maximum activity about 10% that achieved with Mg^{2+}.

Temperature effects. Both L-isoleucine and L-valine bind more strongly to isoleucyl-tRNA-synthetase at 25° than at 10° (Table 6). This suggests that an important cohesive force in the complexes comes from "hydrophobic" interactions. We can estimate from our data that the entropy change on binding L-isoleucine to the enzyme at 25° is 100 ± 70 entropy units. It is unlikely that this kind of binding is a common feature of all amino acid activating enzymes, and even with isoleucyl-tRNA-synthetase electrostatic forces probably play a part too (the $-NH_3^+$ group has been shown to be essential for binding of L-tyrosine to tyrosine-tRNA-synthetase [23]).

PP_i binding. Others have shown that PP_i inhibits amino acid activation by isoleucyl-tRNA-synthetase [2,3]. The results in Table 8 suggest that the reason is that PP_i prevents the binding of ATP.

Comparison with other published data

Protection experiments. Various protection experiments involving isoleucyl-tRNA-synthetase have been described. The processes involved have been inactivation by heat and trypsin [7], tritium exchange [24], and chemical reaction (with e.g., N-ethylmaleimide) [13], but in no case has a detailed and systematic study of the protection effects been made. The extent of protection exerted under given conditions varies because of the different modes of action on the enzyme which are involved. Where comparisons can be made our results correlate with those of others.

Binding constants. Binding interactions of isoleucyl-tRNA-synthetase related to the amino acid activation reaction have previously been studied by kinetic methods [2,20,25]. In the most detailed study [2] constants (ϕ) are deduced from the measured kinetics which can then be assigned to ratios of dissociation constants assuming that amino acid activation has a particular kinetic form. The data are compared with ours in Table II. It is apparent that there is good agreement between values for ϕ_2 and ϕ_4, but a difference of 5 to 10-fold in ϕ_1 and ϕ_3.

This may be entirely attributable to the use of different solvent systems, but our results cast doubt on this. Apart from the presence of urea the only difference between solvents is ionic strength. We have shown that K_{Ile} is not much changed when [urea] is varied. If our $K_{equ} = K_{equ}$ (aqueous) it suggests that our $K_{equ} = K_{equ}$ (aqueous) as their ratio agrees well with ϕ_2 (aqueous) (Table 11).

We have therefore looked more closely at the kinetic results to see how sensitive reaction rates would be to changes in dissociation constants of the order required by our data. There is a problem in understanding the kinetics which derives from the effects of $[Mg^{2+}]$. The $[Mg^{2+}]$ values are not quoted, but are said to be optimal for catalysis. Subsequently it has been shown that optimum $[Mg^{2+}]$ values vary with the concentrations of other substrates, and that the fraction of a substrate complexed with Mg^{2+} varies even under the optimum conditions [5]. We have shown that both ATP and PP_i bind to isoleucyl-tRNA-synthetase in the absence of Mg^{2+}. Thus under given conditions the amounts of ATP and PP_i available for binding to the enzyme are different from the amounts able to participate in catalysis.

In spite of this difficulty we have considered a series of total substrate concentrations (Table 12) which cover the range of values for which data have been shown [2]. The differences in 1/V values calculated for the 2 sets of ϕ-values fall outside the inaccuracies of kinetic measurements. Almost all the discrepancy is due to the ϕ_1 term. Under none of the conditions tested does the ϕ_3 term account for more than 1.2% of the total (using Cole and Schimmel's values) and under most conditions it is very much less. Even using our ϕ_3 value its term never contributes more than 7.5% of the total, and usually it is less than 5%. It seems possible, therefore, that our K_{equ} value is close to K_{equ} (aqueous) but for this to be so K_{ATP} (aqueous) must be $\sim 10^{-3}$ M.

Reaction intermediate. Cole and Schimmel have defined a constant, K_X, by $K_X = [\text{Enzyme-AMP-Ile}][PP_i]/[X]$, where X is an intermediate formed by isoleucyl-tRNA-synthetase and bound substrates prior to the formation of L-isoleucyladenylate [2]. An obvious candidate for X is the complex we have called Enzyme-Ile-ATP (Table 10). On this assumption we calculate a value for K_X of $\sim 10^{-2}$ M. The value deduced from kinetics is 5×10^{-3} M.

Mechanism of $tRNA^{Ile}$ charging. We can make one comment on the discussion whether tRNA charging is a concerted reaction or one which proceeds via the L-isoleucyladenylate intermediate. An argument in favour of the concerted reaction has been that inhibition of PP_i exchange by hydroxylamine is far greater than can be explained by competition between hydroxylamine and PP_i for the adenylate [3]. The concentrations of hydroxylamine used are similar to those of urea in our experiments, and the reduction in activity is comparable. Urea is a weaker nucleophile than hydroxylamine and is not expected to react either with L-isoleucyladenylate or in a concerted reaction catalysed by the enzyme. It thus seems possible that inhibition of PP_i exchange by hydroxylamine is not directly related to its ability to react chemically under the same conditions.

Conformation changes. It is possible that isoleucyl-tRNA-synthetase changes conformation during catalysis [1,26]. Our data provides a body of circumstantial evidence that no such changes which involve major reorganisation of the protein occur during the amino acid activating sequence. No changes in the fluorescence properties

(including polarisation) of the enzyme were observed (cf., the optical rotatory dispersion experiments of Vail and DeLuca [24]). No otherwise inexplicable changes in ligand binding occurred either on altering physical conditions or on binding a second ligand.

Yarus and Berg showed evidence which suggests that interaction with $tRNA^{Ile}$ induces a conformation change in the enzyme, though their measurements were at 17° and pH 5.5 far from optimum conditions for enzyme activity. Baldwin and Berg found that $tRNA^{Ile}$ induces the hydrolysis of L-valyladenylate bound to isoleucyl-tRNA-synthetase, but again the measurement was at low pH [25]. Perhaps the conformational rigidity of the enzyme is pH dependent. The finding that PP_i exchange rate is unaffected by the presence of tRNA at pH 7.5 [3] certainly supports the idea that if there is a tRNA induced conformation change in the enzyme at this pH it only occurs in the absence of other substrates.

Evaluation of the fluorescence decay method for measuring dissociation constants

Advantages. The fluorescence decay method for measuring dissociation

constants for the complexes of isoleucyl-tRNA-synthetase has the following advantages: 1) It requires only small quantities of purified enzyme; measurements were easily made with 2×10^{-8} M solutions of isoleucyl-tRNA-synthetase. 2) Observing inactivation by the rate of decay of fluorescence is a continuous measurement, considerably quicker than assaying for enzymic activity at a series of times. 3) The method is readily adapted to use over a range of physical conditions (temperature, pH, ionic strength, etc.); it is not limited to only those conditions under which catalytic activity is retained. 4) In addition to studying binary complexes it is possible to investigate the simultaneous interactions of more than one type of ligand with the enzyme. 5) The experimental technique is extremely simple and apart from a fluorometer requires no complex equipment, or radiochemicals.

Limitations. This method, like many involving physical measurements, requires highly purified enzymes because the fluorescence of protein impurities will interfere. When a ligand's optical properties overlap those of the enzyme it may be impossible to choose conditions for satisfactory observation of enzyme fluorescence. The biggest uncertainty,

however, is whether observations made under mildly denaturing condition are directly relevant to the properties of fully active enzyme. This question has to be answered for each enzyme to which the method is applied. In the case of isoleucyl-tRNA-synthetase we can say the following: 1) Buffer alone, at pH 7.5 or 8.0 and 25°, is a solvent in which enzyme fluorescence and activity decay slowly (Table 5, Fig. 2). Addition of urea increases the rate of the process. 2) The free energies of transfer of various small molecules from water to urea have been measured [16]. It is possible to infer that the standard free energy of transfer of L-isoleucine from water to 2.5 M urea is -100 to -150 cal/mol. This means that dissociation constants of 10^{-3} M or less are unlikely to differ by more than a few percent in the the two solvents. 3) The dissociation constant of Enzyme-Ile does not vary with [urea] (Table 6). 4) The enzyme is active in 2.5 M urea at pH 8.0 but with its specific activity reduced to about 1/3 the value in Tris-HCl at pH 8.0 (Fig. 9). This could be a thermodynamic (binding) effect, a kinetic effect or a combination of both (see below). These facts suggest that dissociation constants measured for the complexes of

isoleucyl-tRNA-synthetase in 2.5 M urea are of the same order of magnitude as those for a true aqueous solution.

The absence of protection by a potential ligand in our experiments does not prove that there is no interaction between it and the enzyme. The results in Table 6, however, show some correlation between strength of binding and maximum protection, sothat it is likely that any complex with a dissociation constant less than 10^{-4} M would have been detected in our experiments. Nor have we found a case where we have failed to see an effect on the rate of decay of fluorescence when there is independent evidence that a complex is formed. We do not know if isoleucyl-tRNA-synthetase is typical in this regard.

Difficulties. There are some problems with the fluorescence decay method. 2.5 M solutions of crystalline urea obtained commerically have a weak fluorescence emission with excitation and emission maxima around 290 nm and 380 nm. The intensity is stable for hours. Whilst it is possible to make measurements at enzyme concentrations high enough for the urea emission to be negligible in comparison it was found satisfactory

to use more dilute solutions in which the contribution of the urea fluorescence made up about half the initial total. The constant contribution is included in $F_{t=\infty}$ and has no effect on the way rates are calculated.

The excitation and emission wavelengths used in our fluorescence measurements are close enough for Raman scattered light to become a problem if wide slits are used. It is thus best to minimize slit widths by using high gain on the fluorometer.

Although rates of fluorescence decay were quite reproducible on a given day there was more variation ($\pm$ 40%) over a long period. Similar variations show up in the overall extent of reaction ($F_{t=\infty} / F_{t=0}$ = 0.2 to 0.4). Possible reasons for this are slight changes in [urea], temperature, and pH. Some samples may have contained enzyme which was already denatured, and thus gave a higher $F_{t=\infty} / F_{t=0}$ than pure native enzyme. However, none of these variations should cause major differences in dissociation constants measured for a given ligand on different occasions.

Precision. Most of the dissociation constants we have measured have a precision better than ±50% as estimated both from the coincidence of equivalent experiments (Table 6) and from statistical analysis of the points obtained in a single experiment (Fig. 5). Those measurements in which protection was greatest were the most precise. For a satisfactory measurement of K using our technique the upper limit for the fluorescence decay rate at maximum protection is about 70% of the unprotected rate. Greater precision and measurements with lower total protection might be achieved if higher enzyme concentrations and more highly purified urea solutions were used.

Generality. We have no evidence how general the fluorescence decay method may prove to be. It can only be used with proteins that fluoresce with emission characteristics that change on denaturation, and it fails when a ligand's optical properties are obstructive at the concentrations necessary to achieve binding. It is known, however, that urea frequently affects the quantum yield of protein fluorescence, though not always by reducing it [17]. Free energies of protein denaturation in water are often quite small (about +10 Kcal/mol [18])

so that it can be predicted that the equilibrium between native and denatured forms will be sensitive to binding interactions with dissociation constants of 10^{-3} M or less. A denaturing agent like urea functions by lowering the free energy of denaturation further [18], so it should usually be possible to find a concentration range where $\Delta G^{o}_{denaturation}$ is small. Under these conditions the equilibrium between native and denatured states, and the kinetics of interconversion between them will probably be sensitive to the binding of ligands.

These observations imply that the rate of change of fluorescence method may prove quite widely applicable, though it is redundant for studying those interactions in which ligand binding alone causes measurable changes in the fluorescence parameters of the protein.

CONCLUSION

Four objectives in this work were set out in the introduction. We conclude 1) that isoleucyl-tRNA-synthetase does form complexes as proposed by Cole and Schimmel [2] though some of their dissociation constants may have to be modified; 2) 3 mM Mg^{2+} does not critically

affect binding of any of the ligands which interact with the enzyme;

3) the binding interactions are not very sensitive to ionic strength but the temperature dependence of amino acid binding suggests that "hydrophobic" forces are important in stabilising the complexes;

4) the amino acid activation reaction depends crucially on the stabilisation of L-isoleucyl-adenylate by binding to the enzyme. The catalysis does not seem to require major conformation changes in the enzyme. The effect of Mg^{2+} on the enzyme catalysed reaction may be largely to reduce electrostatic repulsion between ATP and the carboxyl group of L-isoleucine.

Mrs. Marie Hebert and Mrs. Ann Orme gave skilled assistance by preparing isoleucyl-tRNA-synthetase and performing the assays of the activity. We are most grateful to Dr. P. R. Schimmel for the copy of a manuscript prior to its publication and to Dr. K. Sauer for his helpful comments after reading this paper. This work was supported by the U. S. Atomic Energy Commission.

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Table 1

Effect of temperature on the fluorescence intensity of isoleucyl-tRNA-synthetase

Excitation was at 280 nm. Emission was observed at 340 nm. The buffer in A was 0.02 M potassium phosphate (pH 7.5) and in B and C it was 0.1 M Tris-HCl (pH 8.0). Enzyme concentrations were 10 µg/ml.

The numbers given for A are averages of the values measured heating from 4° to 35° and then cooling to 4° (because there is an appreciable rate of fluorescence decay at 35°).

	Temperature (°)					
	4	10	18	20	25	35
A. Emission intensity of enzyme alone (relative to intensity at 4°= 100)	100			82		70
B. Emission intensity of enzyme alone (relative to intensity at 10°= 100)		100	89		78	

Table 1 (Continued)	4	10	18	20	25	35
C. Emission intensity of enzyme + 10 mM magnesium acetate (relative to intensity of B at 10°=100)		85	69		59	
$\frac{\text{Intensity of run C}}{\text{Intensity of run B}}$		85	77		75	

Table 2

Polarisation of fluorescence values for isoleucyl-tRNA-synthetase

Emission was observed at 340 nm or 345 nm with a band width of 16 nm to 22 nm. The excitation band width was 5 nm to 7 nm. Buffer A was 0.02 M potassium phosphate (pH 7.5), buffer B was 0.1 M Tris-HCl (pH 8.0) and buffer C was 0.05 M potassium phosphate (pH 5.5). Temperature was 10°. Enzyme concentration was 10 µg/ml. The polarisation values have a maximum error of ± 0.01.

Buffer system	Polarisation of fluorescence exciting at:			
	260 nm	270 nm	280 nm	290 nm
A	0.069	0.067	0.054	0.056
A+0.5 mM L-isoleucine	0.066	0.065	0.056	
A+0.3 M NaCl	0.067	0.064	0.053	
A+0.6 mM magnesium acetate	0.066	0.062	0.059	
A+1.0 mM magnesium acetate			0.058	
A+1.0 mM Na_2ATP			0.053	0.050
A+1.0 mM Na_2ATP + 1.0 mM magnesium acetate			0.056	0.050

Table 2 (Continued

	260 nm	270 nm	280 nm	290 nm
A+1.0 mM Na_4PP_i			0.055	0.056
A+1.0 mM Na_4PP_i + 1.0 mM magnesium acetate			0.055	0.059
B	0.084	0.079	0.068	
B+10 mM magnesium acetate	0.089	0.083	0.074	
C				0.070

Table 3

Species which do not perturb the fluorescence emission of isoleucyl-tRNA-synthetase

The buffer was 0.02 M potassium phosphate (pH 7.5). Enzyme concentrations were 10 µg/ml. Temperature was 10°.

5.0 mM	L-isoleucine
5.0 mM	L-valine
5.0 mM	L-leucine
1.0 mM	glutathione
0.3 M	NaCl
0.6 mM	magnesium acetate
1.0 mM	Na_4PP_i
1.0 mM	Na_4PP_i + 1.0 mM magnesium acetate

Table 4

Effect of ionic strength on the rate of decay of isoleucyl-tRNA-synthetase fluorescence intensity in 2.5 M urea

Excitation was at 280 nm. Emission was observed at 340 nm. Enzyme concentration was 3.5 μg/ml. Temperature was 25°. All runs were at pH 8.0. The experimental technique is described in the text.

Buffer composition	Relative rate of decay of fluorescence
0.06 M Tris-HCl	108
0.10 M Tris-HCl	100
0.10 M Tris-HCl + 0.5 M KCl	97

Table 5

Dependence of the rates of decay of isoleucyl-tRNA-synthetase fluorescence on urea concentration

Excitation was at 280 nm. Emission was observed at 340 nm. The buffer was 0.02 M potassium phosphate. Enzyme concentration was 3.5 µg/ml. Temperature was 25°.

[urea] (M)	Relative rate of fluorescence decay
2.5	100
1.7	14
0.8	11
0	~5

Table 6

Dissociation constants for binary complexes between isoleucyl-tRNA-synthetase and L-isoleucine, L-valine, ATP, and PP_i

Enzyme concentrations were in the range 2 to 10 µg/ml. The method used, and the accuracy of the results are described and discussed in the text.

Dissociation constant (M)

(The figures in parentheses give the rate of fluorescence decay at maximum protection relative to the unprotected rate)

L-isoleucine	L-valine	Na_2ATP	Na_4PP_i
a) Buffer was 0.1 M Tris-HCl (pH 8.0); temperature was 25°; [urea] = 2.5 M.			
2.5×10^{-5} (30%)	3.0×10^{-4} (40%)	1.0×10^{-4} (55%)	2.5×10^{-4} (65%)
2.5×10^{-5} (40%)		2.0×10^{-4} (65%)	2.0×10^{-4} (65%)
b) Buffer was 0.02 M potassium phosphate (pH 7.6); temperature was 25°; [urea] = 2.5 M.			
3.0×10^{-5} (30%)	1.5×10^{-4} (40%)	1.2×10^{-4} (60%)	5.0×10^{-5} (55%)

Table 6 (Continued)

L-isoleucine	L-valine	Na_2ATP	Na_4PP_i

c) Buffer was 0.1 M Tris-HCL (pH 8.0); temperature was 10°; [urea] = 2.5 M.

	3.0×10^{-4} (10%)	$\sim 10^{-2}$ ($\sim$50%)

d) Buffer was 0.02 M potassium phosphate at pH 7.6; temperature was 10°; [urea] = 2.5 M.

	2.0×10^{-4} (25%)	

e) Temperature was 25°.

	2.0×10^{-5} (40%)	in .02 M potassium phosphate (pH 7.6); [urea] = 0.8 M.
	4.0×10^{-5} (30%)	in .02 M potassium phosphate (pH 7.6); [urea] = 1.7 M.
	1 to 3×10^{-5} (40%)	in 0.1 M Tris-HCl (pH 8.0); [urea] = 1.25 M.

Table 7

Effects of Mg^{2+} and ionic strength on the formation of binary complexes by isoleucyl-tRNA-synthetase

All solutions contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea, and 2 to 5 μg/ml enzyme. Other additions are listed in the Table. Temperature was 25°. The method is described in the text.

		Relative rates of fluorescence decay		
		No addition	3.3 mM magnesium acetate	0.33 M KCl
[L-isoleucine] (M)	0	100	100	97
	$3x10^{-5}$	75	67	66
	$3x10^{-4}$	45	54	43
[Na_2ATP] (M)	0	100	99	97
	10^{-4}	85	76	66
	$3x10^{-4}$	76	58	59
[Na_4PP_i] (M)	0	100	100	100
	$1.7x10^{-4}$	87	93	84
	$1.7x10^{-3}$	70	76	72

Table 8

Rates of decay of the fluorescence of isoleucyl-tRNA-synthetase in the presence of 2 ligands

All solutions contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea, and 2 to 5 µg/ml enzyme. Other additions are listed in the Table. Temperature was 25°. The method is described in the text.

Further additions	Relative decay rate
None	100
1.6 mM L-isoleucine	35
0.3 mM Na_2ATP	65
1.6 mM Na_4PP_i	65
1.6 mM L-isoleucine + 0.3 mM Na_2ATP	25
1.6 mM L-isoleucine + 1.6 mM Na_4PP_i	25
0.3 mM Na_2ATP + 1.6 mM Na_4PP_i	55

Table 9

Effects of magnesium acetate on isoleucyl-tRNA-synthetase in the presence of ATP and L-isoleucine

All solutions contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea, and 5 to 10 µg/ml enzyme. Other additions are listed in the Table. Temperature was 25°. The method is described in the text.

Further additions (mM)			Relative rate of decay of fluorescence
[L-isoleucine]	[ATP]	[Magnesium acetate]	
0	1.0	0	100
Limit [Ile] → ∞	1.0	0	45
1.7	1.0	0	60
1.7	1.0	1.7	90
0.0017	0.01	0	150
0.0017	0.01	1.7	105

Table 10

Standard free energies of formation of some complexes of isoleucyl-tRNA-synthetase at 25°

	Reaction	$\Delta G°$* (Kcal/mol)
1.	Enzyme + Ile → Enzyme-Ile	-7
2.	Enzyme + ATP → Enzyme-ATP	-6
3.	Enzyme-ATP + Ile → Enzyme-ATP-Ile	-6
4.	Enzyme + Ile + ATP → Enzyme-AMP-Ile + PP_i	-9
5.	ATP + Ile → AMP-Ile + PP_i	+2†
6.	Enzyme + AMP-Ile → Enzyme-AMP-Ile	-11 (4-5)

* $\Delta G°$ is calculated from K-values in Table 6 and the text using the relation $\Delta G° = -RT \ln K$.

† Estimated from data in ref. 19.

Table 11

Comparison of equilibrium constant ratios (ϕ) obtained from kinetics [2] and from the measurements in Table 6 and Fig. 8

ϕ (as defined in ref. 2)	ϕ (as measured in ref. 2) (M)	ϕ (calculated from this work) (M)
$\phi_1 = K_{equ}/K_{ATP}$	2.2×10^{-3}	2×10^{-2}
$\phi_2 = K_{equ}/K_{Ile}$	8.25×10^{-2}	8×10^{-2}
$\phi_3 = K_{equ}$	3.3×10^{-7}	2×10^{-6}
$\phi_4 = K_{equ}/K_{PP_i}$	1.1×10^{-2}	1×10^{-2}

Table 12

Substrate concentrations for which reciprocal velocity (1/V) values have been compared

The equation (taken from ref. 2) used in all calculations is

$$\frac{1}{V} \propto \left\{ \frac{1}{[PP_i]} + \frac{\phi 1}{[Ile]} + \frac{\phi 2}{[ATP]} + \frac{\phi 3}{[Ile][ATP]} + \frac{\phi 4[PP_i]}{[Ile][ATP]} + \phi 5 \right\}$$

ϕ_{1-4} are defined and the 2 sets of values used are given in Table 11. ϕ_5 is taken as 500 in each case [2]. All 27 combinations of the following substrate concentrations have been considered:

	L-isoleucine (μM)	ATP (mM)	PP_i (mM)
	10	0.5	2.0
	25	1.0	4.0
	100	4.0	5.0
and also	10	1.0	0.5
	10	1.0	1.0

LEGENDS TO FIGURES

Fig. 1. Sample plot for determining the limiting fluorescence intensity of isoleucyl-tRNA-synthetase in 2.5 M urea ($F_{t=\infty}$). $\Delta F = F_{t=0} - F_t$. The sample contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea and 3 µg/ml enzyme. Temperature was 25°. $F_{t=\infty} = F_{t=0} - \Delta F_{t=\infty}$

Fig. 2. Kinetics of the loss of enzyme activity compared with isoleucyl-tRNA-synthetase fluorescence decay in 2.5 M urea. Solutions contained 0.02 M potassium phosphate (pH 7.6) and 2.5 M urea. Temperature was 25°. o, % activity = $(F_t - F_{t=\infty})/(F_{t=0} - F_{t=\infty}) \times 100$; enzyme concentration = 2 µg/ml. •, Enzyme (10 µg/ml) was incubated at 25° with aliquots removed periodically for assay by PP_i exchange. % activity is $(\text{moles } PP_i \text{ exchanged})_t/(\text{moles } PP_i \text{ exchanged})_{t=0} \times 100$. Δ was an identical run to • except that urea was omitted.

Fig. 3. Fluorescence excitation and emission spectra of isoleucyl-tRNA-synthetase. Solutions contained 0.1 M Tris-HCl at pH 8.0. Temperature was 10°. A and C are excitation and emission spectra of enzyme (10 µg/ml).

Figure legends (Continued)

B and D are excitation and emission spectra of buffer alone. In A and B emission was observed at 340 nm with 7 nm band width. In C and D excitation was at 280 nm with 5 nm band width. The spectra are not corrected for variations in instrument response with wavelength.

Fig. 4. Reproducibility of the measurement of rates of decay of isoleucyl-tRNA-synthetase fluorescence.

$F_{t=0}$ and ΔF are defined in the legend to Fig. 1. Solutions contained 0.02 M potassium phosphate (pH 7.6), 2.5 M urea, and 8 μg/ml enzyme. Temperature was 10°. Excitation and emission wavelengths were 280 nm and 337 nm. o and • are for solutions exactly as described; □ and ■ each contained, in addition, 0.33 mM L-isoleucine.

Fig. 5. Sample double reciprocal plots for determining dissociation constants of some binary complexes of isoleucyl-tRNA-synthetase.

These graphs correspond to data in the first lines of Table 6a, in which experimental conditions are described. $D = R_{[L]=0} - R_{[L]}$: the units in which D is plotted, when multiplied by 1.39, give D in

Figure legends (Continued)

min^{-1}. Direct comparison between ordinate values in the different graphs is not meaningful because of variations in $R_{[L]=0}$. Accuracy of the measurements is discussed in the text.

Fig. 6. Double reciprocal plot for the binding of L-isoleucine to isoleucyl-tRNA-synthetase in the presence of 1.0 mM ATP.

All solutions contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea, 1.0 mM Na_2ATP and 3 µg/ml enzyme. Temperature was 25°. D was defined in the legend to Fig. 5.

Fig. 7. Double reciprocal plot for determining K_{eqn} for the PP_i exchange reaction catalysed by isoleucyl-tRNA-synthetase.

All solutions contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea, 2.5 mM magnesium acetate, and 6 µg/ml enzyme. Temperature was 25°. D was defined in the legend to Fig. 5. o, L = L-isoleucine; •, L = Na_2ATP.

Fig. 8. Effect of magnesium acetate on the rate of fluorescence decay of isoleucyl-tRNA-synthetase under conditions which permit catalysis.

All solutions contained 0.1 M Tris-HCl (pH 8.0), 2.5 M urea, 5×10^{-6} M

Figure Legends (Continued)

Na_2ATP, 10^{-6} M L-isoleucine and 5 μg/ml enzyme. Temperature was 25°. □, △, ○ and ■ denote four independent sets of measurements (each normalised to give a decay rate of 100 when $[Mg^{2+}] = 0$): ● gives average decay rates.

Fig. 9. Effect of 2.5 M urea on the specific activity of isoleucyl-tRNA-synthetase.

The PP_i exchange assay was performed under the usual conditions at 25° (see text) with ○ L-isoleucine or □ L-valine as substrate. The runs with solid points (● L-isoleucine and ■ L-valine) were similar except that the incubation mixture contained 2.5 M urea.

Fig. 10. Dependence of the specific activity of isoleucyl-tRNA-synthetase on $[MgCl_2]$.

The composition of incubation mixtures is as described in the text except for varying $[MgCl_2]$. Enzyme was 4 μg/ml. Temperature was 25°. Incubation times were 15 min. Specific activities for both L-isoleucine (●) and L-valine (○) have been normalized to 100 at 10^{-2} M $MgCl_2$.

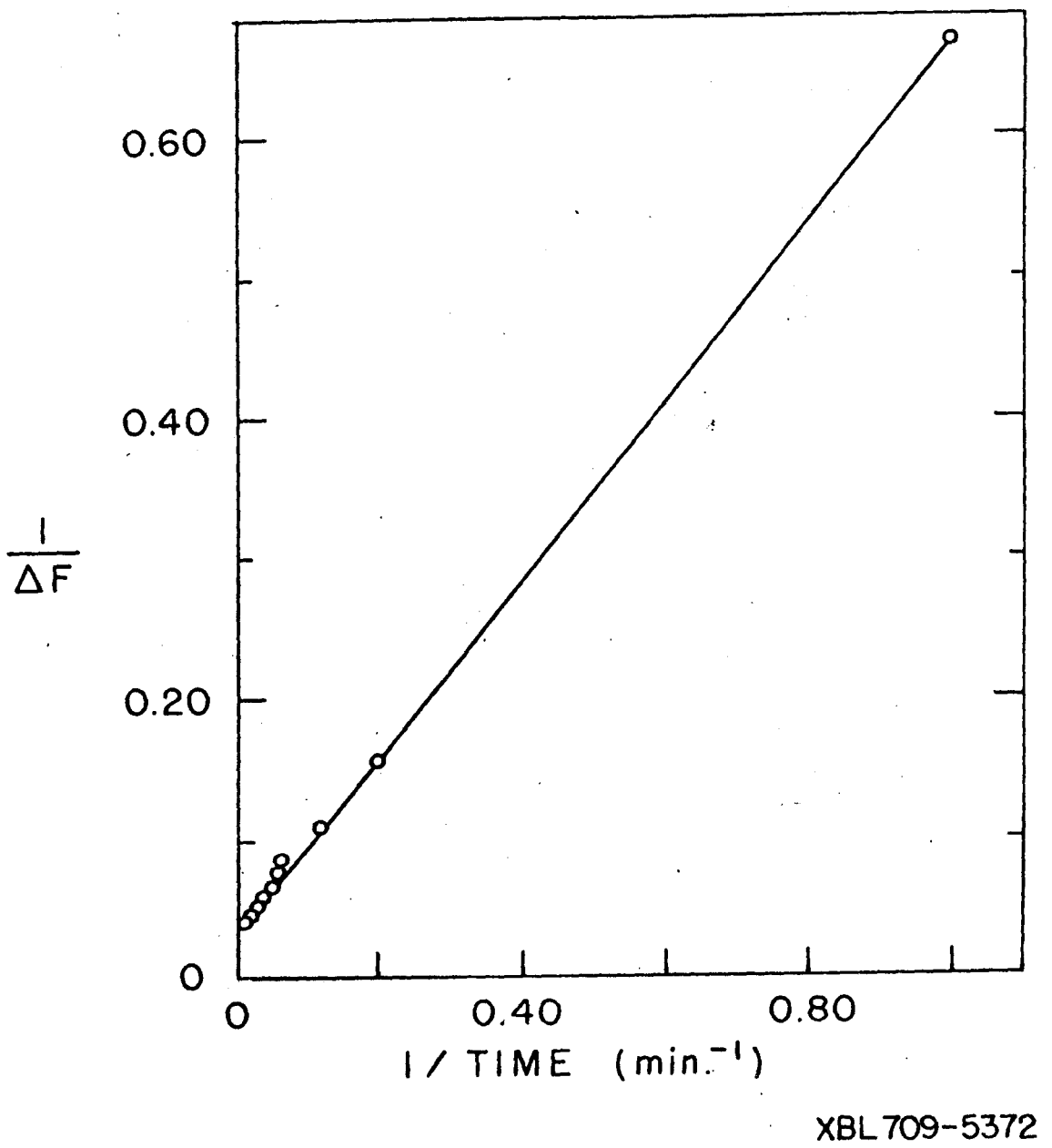
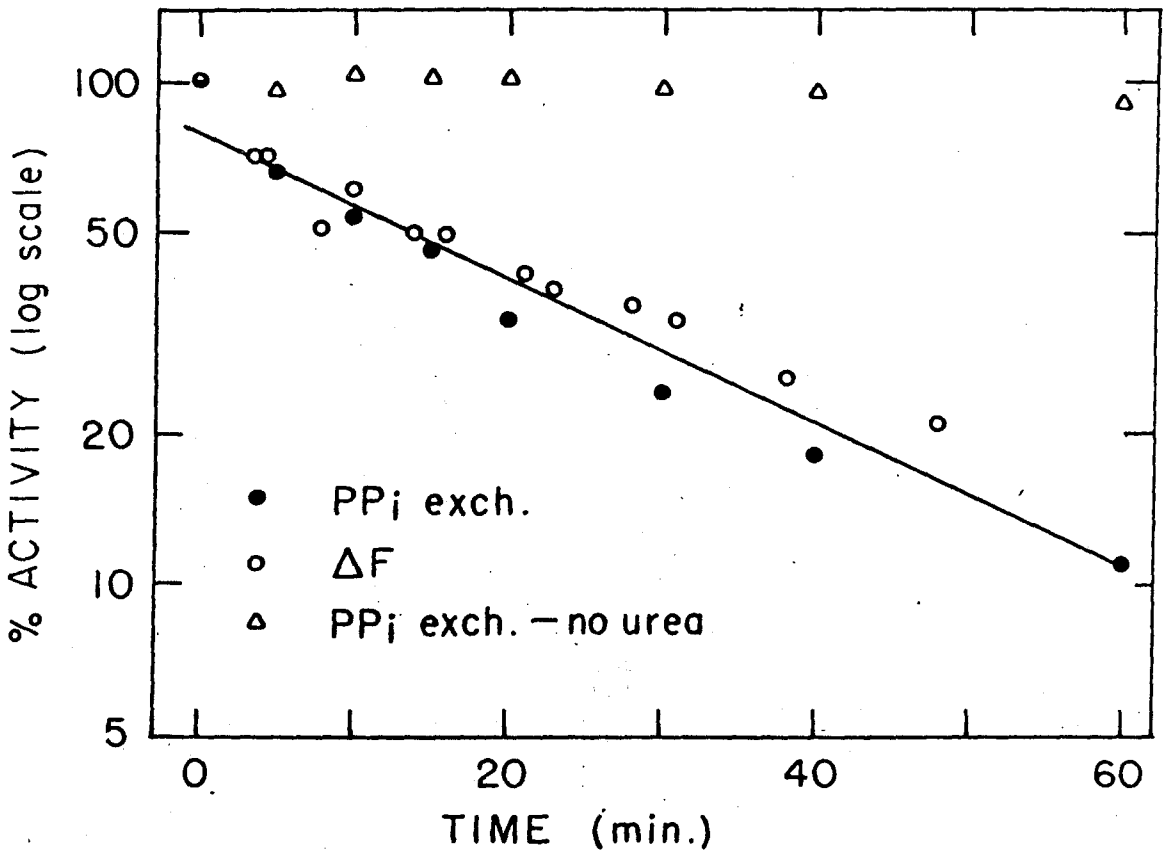
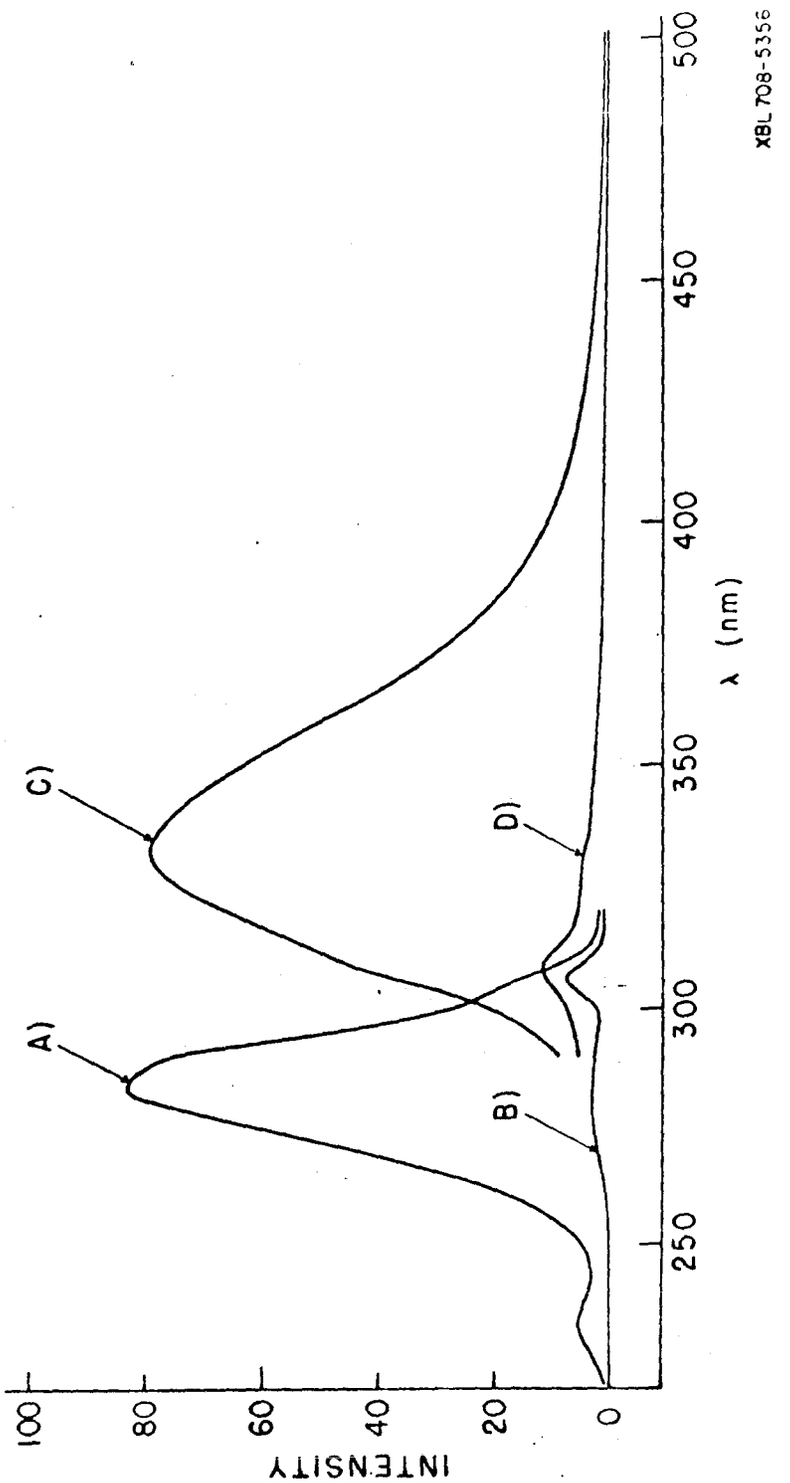


Fig. 1



XBL709-5370

Fig. 2


INTENSITY
100
80
60
40
20
0
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400
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λ (nm)
A)
B)
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D)
XBL 708-5356

Fig. 3

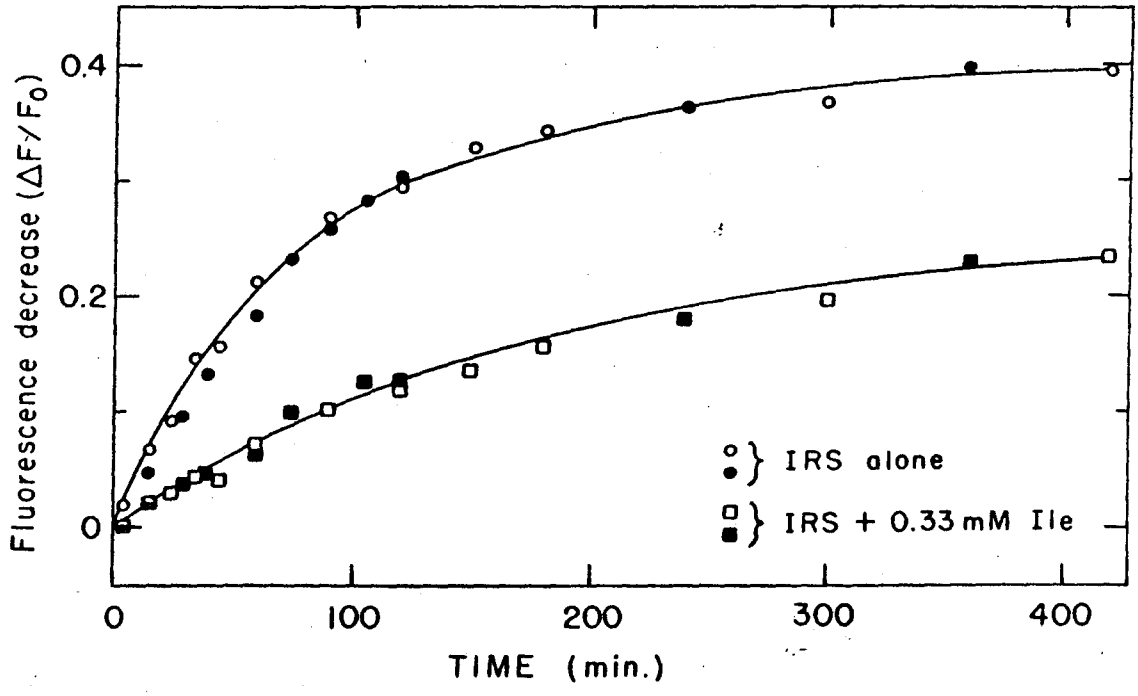
Fluorescence decrease (ΔF/F0)
0.4
0.2
0
0
100
200
300
400
TIME (min.)
IRS alone
IRS + 0.33 mM Ile
XBL 709-5373

Fig. 4

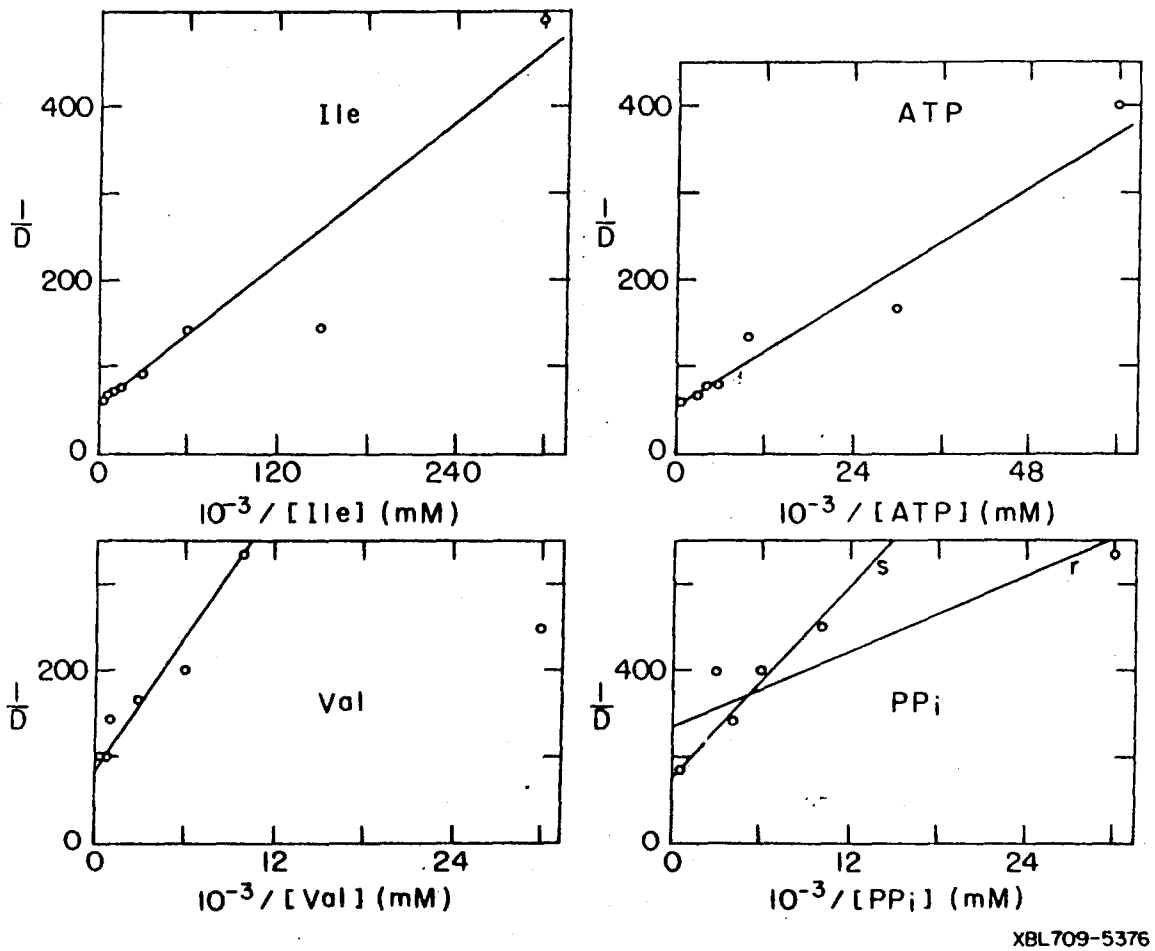
Ile
400
200
0
0
120
240
10^{-3} / [Ile] (mM)
ATP
400
200
0
0
24
48
10^{-3} / [ATP] (mM)
Val
200
0
0
12
24
10^{-3} / [Val] (mM)
PPi
s
r
400
0
0
12
24
10^{-3} / [PPi] (mM)
1/D
XBL709-5376

Fig. 5

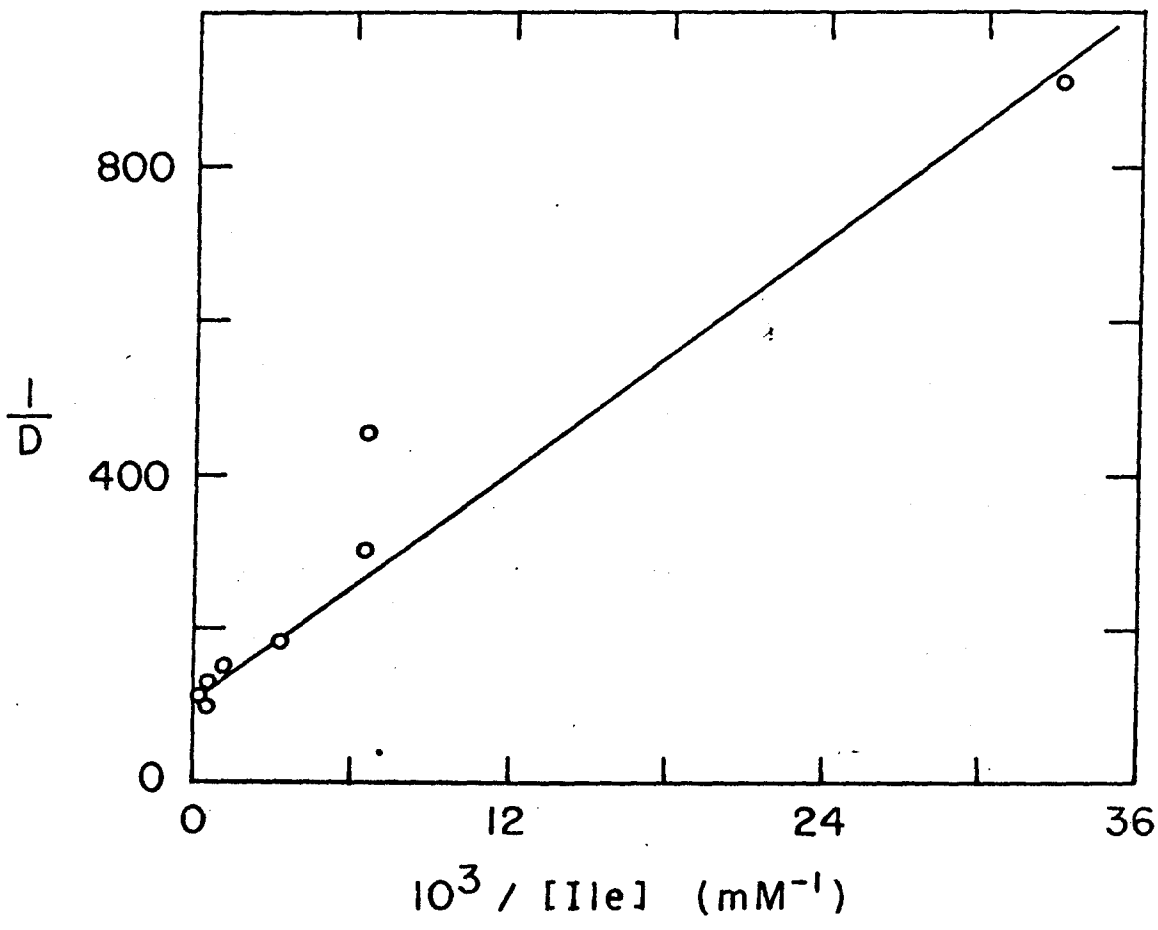

Fig. 6

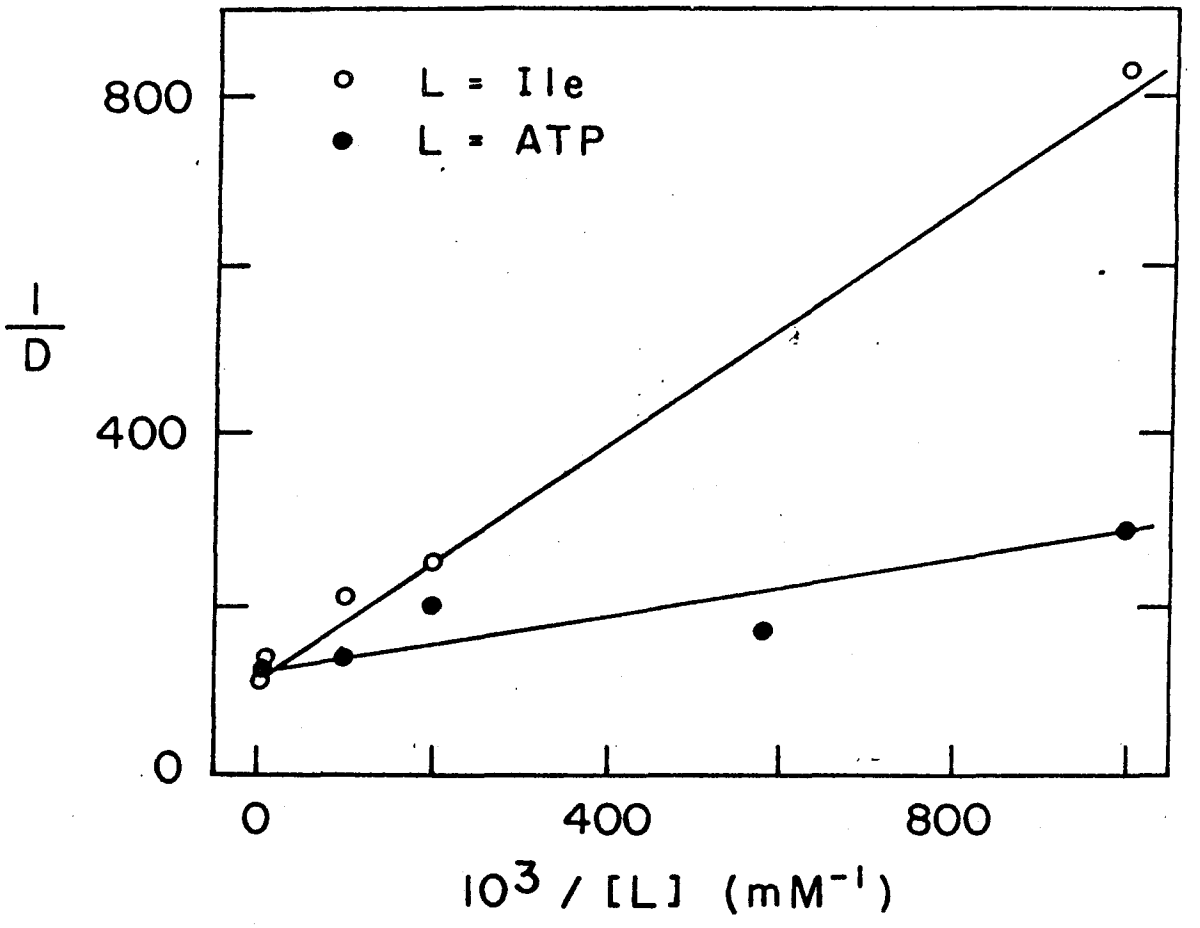

Fig. 7

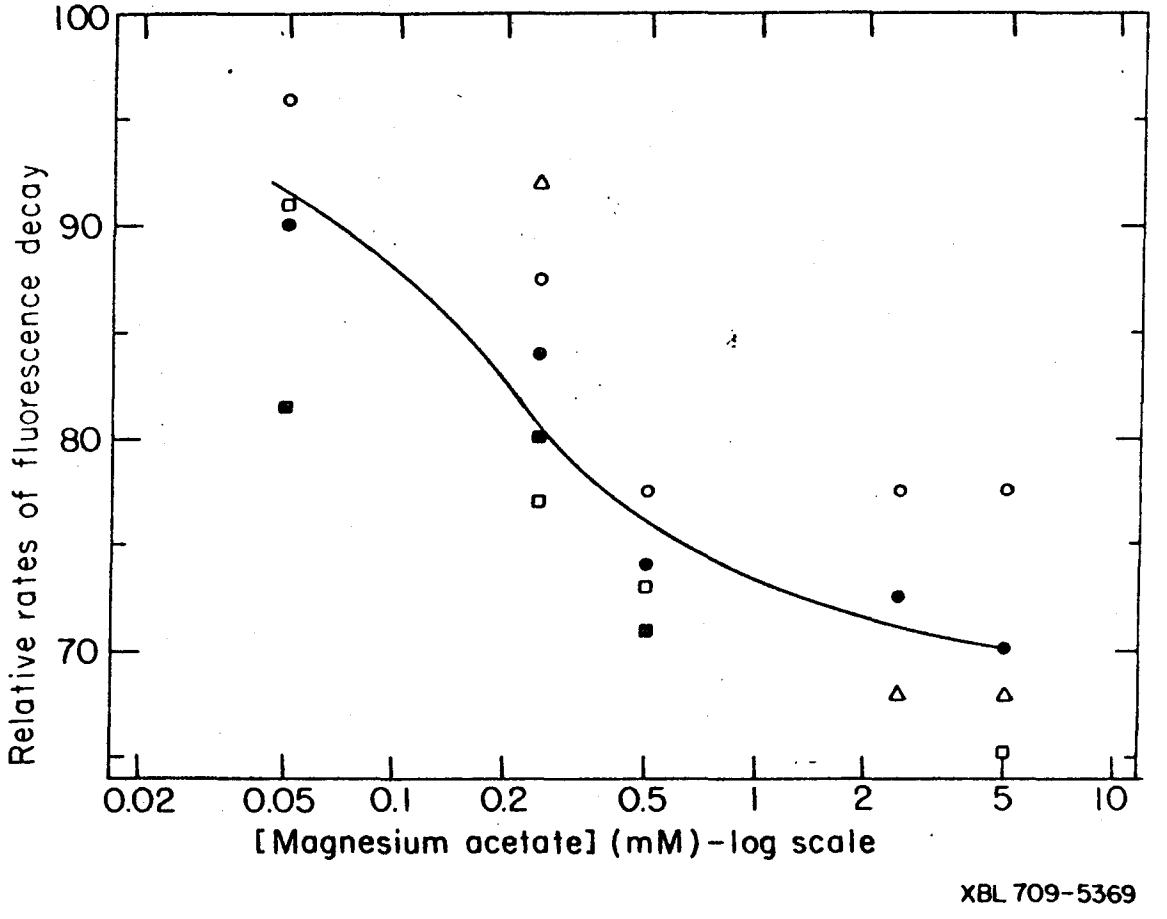
100
90
80
70
Relative rates of fluorescence decay
0.02
0.05
0.1
0.2
0.5
1
2
5
10
[Magnesium acetate] (mM)-log scale
XBL 709-5369

Fig. 8

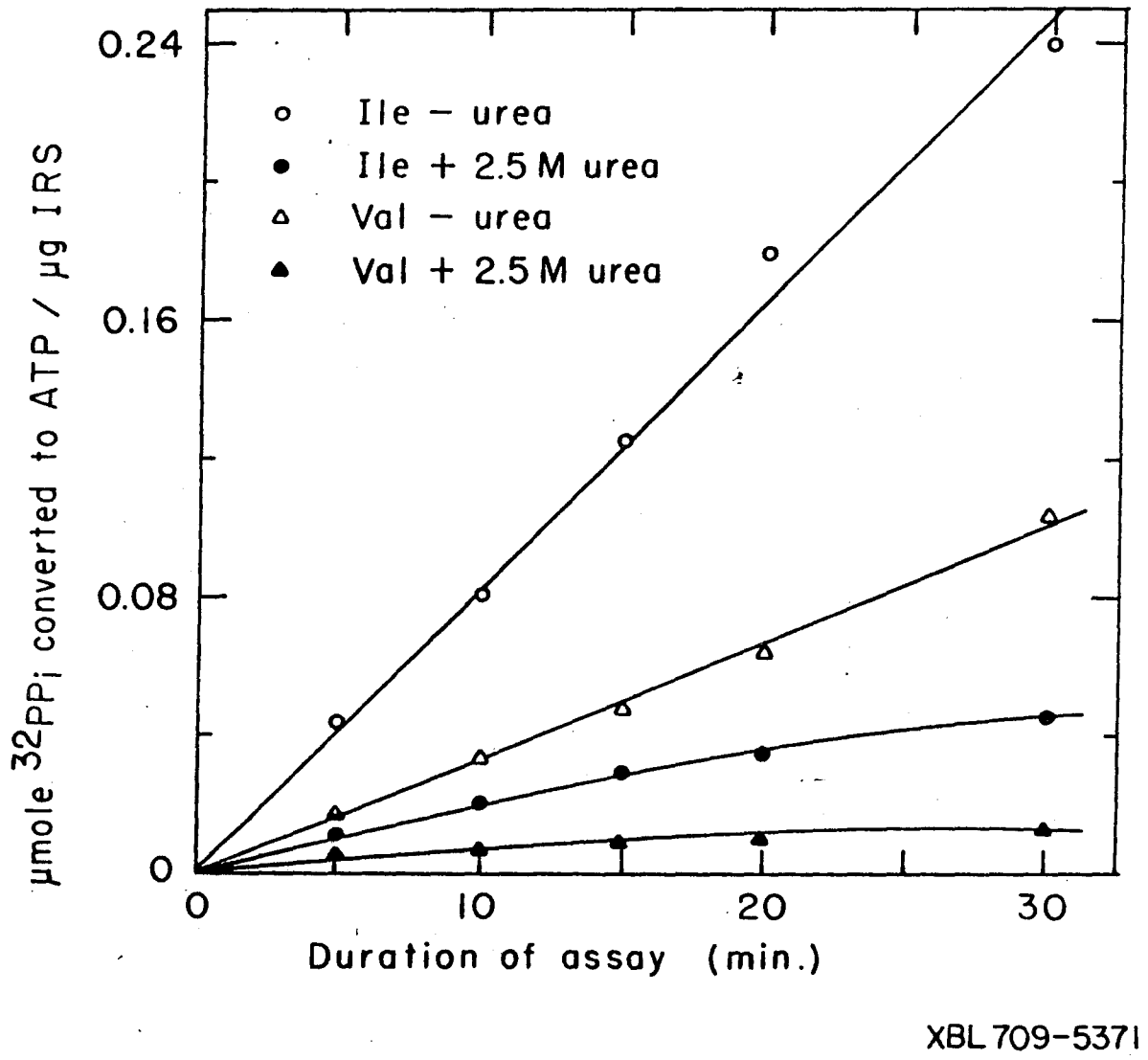
0.24
0.16
0.08
0
0
10
20
30
Ile − urea
Ile + 2.5 M urea
Val − urea
Val + 2.5 M urea
μmole 32PPi converted to ATP / μg IRS
Duration of assay (min.)
XBL 709-5371

Fig. 9

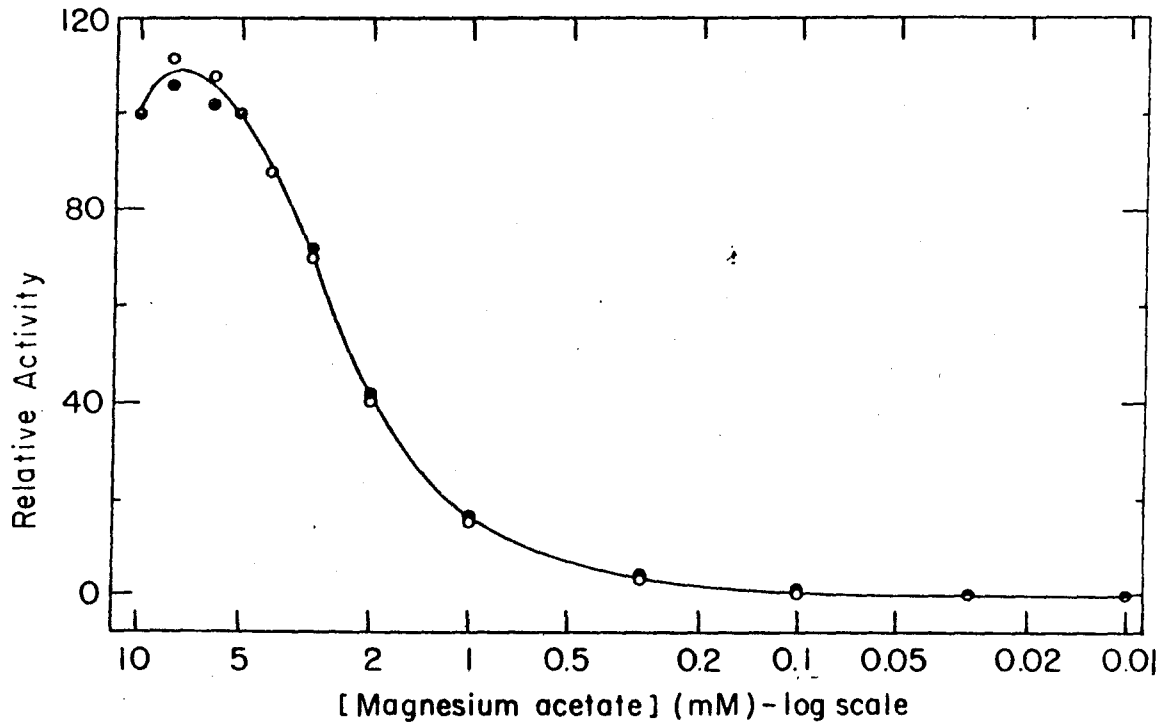
120
80
40
0
Relative Activity
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
[Magnesium acetate] (mM) - log scale
XBL709-5368

Fig. 10

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